Method for the production of ergosta-5,7-dienol and/or its biosynthetic intermediates and/or metabolites in transgenic organisms

The present invention relates to a method for the production of ergosta-5,7-dienol and/or its biosynthetic intermediates and/or metabolites by culturing genetically modified organisms, and to the genetically modified organisms, in particular yeasts, themselves.

Ergosta-5,7-dienol and its biosynthetic intermediates of the sterol metabolism, such as, for example, farnesol, geraniol, squalene and lanosterol and zymosterol, and its biosynthetic metabolites of the sterol metabolism, for example in mammals, such as, for example, campesterol, pregnenolone, 17-OH-pregnenolone, progesterone, 17-OH-progesterone, 11-deoxycortisol, hydrocortisone, deoxycorticosterone or corticosterone, are compounds of high economical value.

Ergosta-5,7-dienol may act as starting compound for the preparation of steroid hormones via biotransformations, chemical synthesis or biotechnological production.

Hydrocortisone has a weak glucocorticoid effect and is a sought-after starting compound for the synthesis of active ingredients with a highly antiinflammatory, abortive or antiproliferative effect.

Squalene is used as building block for the synthesis of terpenes. In its hydrogenated form, it is used as squalane in dermatology and cosmetics, and in its various derivatives as constituent of skincare and haircare products.

Other economically utilizable substances are sterols, such as zymosterol and lanosterol, lanosterol being a pivotal raw material and synthetic material for the chemical synthesis of saponins and steroid hormones. Owing to its good skin penetration and spreading properties, lanosterol is used as emulsion auxiliary and active ingredient for skin creams.

An economical method for the production of ergosta-5,7-dienol and/or its biosynthetic intermediates and/or metabolites is therefore of great importance.

Methods which are particularly economical are biotechnological methods exploiting natural organisms or organisms optimized by means of genetic modification which produce ergosta-5,7-dienol and/or its biosynthetic intermediates and/or metabolites.

The genes of the ergosterol metabolism in yeast are largely known and cloned, such as, for example,

nucleic acids encoding an HMG-CoA reductase *(HMG)*(Bason M.E. et al,(1988) Structural and functional conservation between yeast and human 3-hydroxy-3-methylglutaryl coenzyme A reductases, the rate-limiting enzyme of sterol biosynthesis. Mol Cell Biol 8:3797-3808,

the nucleic acid encoding a truncated HMG-CoA reductase *(t-HMG)*(Polakowski T, Stahl U, Lang C.(1998) Overexpression of a cytosolic hydroxymethylglutaryl-CoA reductase leads to squalene accumulation in yeast. Appl Microbiol Biotechnol. Jan; 49(1):66-71,

the nucleic acid encoding a lanosterol C14-demethylase *(ERG11)* (Kalb VF, Loper JC, Dey CR, Woods CW, Sutter TR (1986) Isolation of a cytochrome P-450 structural gene from *Saccharomyces cerevisiae*. Gene 45(3):237-45,

the nucleic acid encoding a squalene epoxidase *(ERG1)* (Jandrositz, A., et al (1991) The gene encoding squalene epoxidase from *Saccharomyces cerevisiae:* cloning and characterization. Gene 107:155-160 and

nucleic acids encoding a squalene synthetase *(ERG9)* (Jennings, S.M., (1991): Molecular cloning and characterization of the yeast gene for squalene synthetase. Proc Natl Acad Sci USA. Jul15;88(14):6038-42).

There are furthermore known processes which aim at increasing the content in specific intermediates and catabolites of the sterol metabolism in yeasts and fungi.

It is known from T. Polakowski, Molekularbiologische Beeinflussung des Ergosterolstoffwechsels der Hefe *Saccharomyces cerevisiae* [Molecular-biological effects on the ergosterol metabolism of the yeast *Saccharomyces cerevisiae*], Shaker Verlag Aachen, 1999, pages 59 to 66, that increasing the expression rate of HMG-CoA reductase leads to a slightly increased content in early sterols, such as squalene, while the content in later sterols, such as ergosterol, does not change significantly or even has a tendency to decrease.

Tainaka et al., J, Ferment. Bioeng. 1995, 79, 64-66 furthermore describe that the overexpression of ERG11 (lanosterol C14-demethylase) leads to the accumulation of 4,4-dimethylzymosterol, but not ergosterol. In comparison with the wild type, the zymosterol content of the transformant is increased by a factor of 1.1 to 1.47, depend-

ing on the fermentation conditions.

WO 99/16886 describes a method for the production of ergosterol in yeasts which overexpress a combination of the genes *t*HMG, ERG9, SAT1 and ERG1.

EP 486 290 discloses a method for increasing the squalene, zymosterol, ergosta-5,7,-24(28)-trienol and ergosta-5,7-dienol content in yeast by increasing the HMG-CoA reductase expression rate and simultaneously interrupting the metabolic pathway of ergosta-5,7,24(28)-trienol-22-dehydrogenase, hereinbelow also referred to as Δ22-desaturase (ERG5).

However, the disadvantage of this method is that the ergosta-5,7-dienol yield is still not satisfactory.

It is an object of the present invention to provide a further method for the production of ergosta-5,7-dienol and/or its biosynthetic intermediates and/or metabolites with advantageous characteristics, such as a higher product yield.

We have found that this object is achieved by a method for producing ergosta-5,7-dienol and/or its biosynthetic intermediates and/or metabolites in which organisms are cultured which have

a reduced Δ22-desaturase activity and

an increased HMG-CoA reductase activity and

an increased activity of at least one of the activities selected from the group consisting of lanosterol C14-demethylase activity, squalene epoxidase activity and squalene synthetase activity

in comparison with the wild type.

A reduced activity is understood as meaning not only the reduction of the activity, but also the complete elimination of the activity. Accordingly, a reduction of an activity also encompasses a quantitative reduction of the relevant protein in the organism through to a complete absence of the relevant protein, which can be assayed, for example, by a lack of detectability of the relevant enzyme activity or a lack of immunological detectability of the relevant proteins.

Δ22-desaturase activity is understood as meaning the enzyme activity of a Δ22-desaturase.

A Δ22-desaturase is understood as meaning a protein with the enzymatic activity of converting ergosta-5,7-dienol into ergosta-5,7,22,24-tetraen-3β-ol.

Accordingly, Δ22-desaturase activity is understood as meaning the amount of ergosta-5,7-dienol converted, or the amount of ergosta-5,7,22,24-tetraen-3β-ol formed, by the protein Δ22-desaturase within a specific period of time.

Thus, in the case of reduced Δ22-desaturase activity in comparison with the wild type, the amount of ergosta-5,7-dienol converted, or the amount of ergosta-5,7,22,24-tetraen-3β-ol formed, by the protein Δ22-desaturase within a specific period of time is reduced in comparison with the wild type.

The Δ22-desaturase activity is preferably reduced to at least 90%, more preferably to at least 70%, more preferably to at least 50%, more preferably to at least 30%, even more preferably by at least 10%, even more preferably by at least 5%, in particular to 0% of the Δ22-desaturase activity of the wild type. Especially preferred is, accordingly, the eliminination of the Δ22-desaturase activity in the organism.

The Δ22-desaturase (ERG5) activity can be determined as described hereinbelow:

Various concentrations of ergosta-5,7-dienol, isolated from *S. cerevisiae Erg5* mutants (Parks et al, 1985. Yeast sterols.yeast mutants as tools for the study of sterol metabolism. Methods Enzymol. 111:333-346) and 50 μg of dilauroylphosphatidylcholin are mixed and sonicated until a white suspension forms. Processed microsomes are added (1 ml)(3 mg/ml protein). NADPH (final concentration 1 mM) is added to the test mixture in order to start the enzyme reaction. The mixture is incubated for 20 minutes at 37°C. The reaction is stopped by addition of 3 ml of methanol, and sterols are hydrolyzed by addition of 2 ml 60% (wt/vol) KOH in water. The mixture is incubated for 2 hours at 90°C. After cooling, the mixture is extracted three times with 5 ml of hexane and concentrated by evaporation on a rotary evaporator. The sterols are subsequently silylated with bis(trimethylsilyl)trifluoroacetamide (50 μl in 50 μl of toluene) for one hour at 60°C. The sterols are analyzed by gas chromatography/mass spectroscopy (GC-MS) (for example Model VG 12-250 gas chromatograph-mass spectrometer; VG Biotech, Manchester, United Kingdom). The resulting Δ22-desaturated intermediate can be identified as a function of the amount of substrate employed. Microsomes which

are not incubated with substrate act as reference.

This method is a modification of the method described in Lamb et al: Purification, reconstitution, and inhibition of cytochrome P-450 sterol delta22-desaturase from the pathogenic fungus Candida glabrata. Antimicrob Agents Chemother. 1999 Jul;43(7):1725-8.

The Δ22-desaturase activity can be reduced independently by different cytological mechanisms, for example by inhibiting the corresponding activity at the protein level, for example by addition of inhibitors of the enzymes in question, or by reducing the gene expression of the corresponding nucleic acids encoding a Δ22-desaturase in comparison with the wild type.

In a preferred embodiment of the method according to the invention, the Δ22-desaturase activity is reduced in comparison with the wild type by reducing the gene expression of the corresponding nucleic acids encoding a Δ22-desaturase.

Reducing the gene expression of the nucleic acids encoding a Δ22-desaturase in comparison with the wild type can likewise be effected in various ways, for example by

a) introducing nucleic acid sequences which can be transcribed into an antisense nucleic acid sequence which is capable of inhibiting the Δ22-desaturase activity, for example by inhibiting the expression of endogenous Δ22-desaturase activity,

b) overexpressing homologous Δ22-desaturase nucleic acid sequences, which lead to cosuppression,

c) introducing nonsense mutations into the endogen by introducing RNA/DNA oligonucleotides into the organism,

d) introducing specific DNA-binding factors, for example factors of the zinc finger transcription factor type, which bring about a reduced gene expression, or

e) generating knock-out mutants, for example with the aid of T-DNA mutagenesis or homologous recombination.

In a preferred embodiment of the method according to the invention, the gene expression of the nucleic acids encoding a Δ22-desaturase is reduced by generating knock-out mutants, especially preferably by homologous recombination.

Accordingly, it is preferred to use an organism without a functional Δ22-desaturase gene.

In a preferred embodiment, the generation of knock-out mutants, that is to say the deletion of the target locus Δ22-desaturase gene, is carried out simultaneously with the integration of an expression cassette comprising at least one of the nucleic acids described hereinbelow, encoding a protein whose activity is being increased in comparison with the wild type, by homologous recombination.

To this end, it is possible to use nucleic acid constructs which, in addition to the expression cassettes described hereinbelow comprising promoter, coding sequence and, if appropriate, terminator, and in addition to a selection marker described hereinbelow, comprise, at the 3' and 5' end, nucleic acid sequences which are identical to nucleic acid sequences at the beginning and at the end of the gene to be deleted.

Once selection has taken place, it is preferred to remove the selection marker again by means of recombinase systems, for example by loxP signals at the 3' and 5' end of the selection marker, using a Cre recombinase (Cre-LoxP system).

In the preferred organism Saccharomyces cerevisiae, the Δ22-desaturase gene denotes the gene ERG5 (SEQ. ID. NO. 1). SEQ. ID. NO. 2 constitutes the corresponding Saccharomyces cerevisiae Δ22-desaturase (Skaggs, B.A. et al,: Cloning and characterization of the Saccharomyces cerevisiae C-22 sterol desaturase gene,encoding a second cytochrome P-450 involved in ergosterol biosynthesis, Gene.1996 Feb22;169(1):105-9.).

HMG-CoA reductase activity is understood as meaning the enzyme activity of an HMG-CoA reductase (3-hydroxy-3-methylglutaryl-coenzyme A reductase).

An HMG-CoA reductase is understood as meaning a protein with the enzymatic activity of converting 3-hydroxy-3-methylglutaryl-coenzyme A into mevalonate.

Accordingly, HMG-CoA reductase activity is understood as meaning the amount of 3-hydroxy-3-methylglutaryl-coenzyme A converted, or the amount of mevalonate formed, by the protein HMG-CoA reductase within a specific period of time.

Thus, in the case of an increased HMG-CoA reductase activity in comparison with the wild type, the amount of 3-hydroxy-3-methylglutaryl-coenzyme A converted, or the amount of mevalonate formed, by the protein HMG-CoA reductase within a specific period of time is increased in comparison with the wild type.

Preferably, this increase in the HMG-CoA reductase activity amounts to at least 5%, more preferably to at least 20%, more preferably to at least 50%, more preferably to at least 100%, even more preferably to at least 300%, especially preferably to at least 500%, in particular to at least 600% of the HMG-CoA reductase activity of the wild type.

The HMG-CoA reductase activity is determined as described in Th. Polakowski, Molekularbiologische Beeinflussung des Ergosterolstoffwechsels der Hefe Saccharomyces cerevisiae, Shaker-Verlag, Aachen 1999, ISBN 3-8265-6211-9.

According to this reference, 10^9 yeast cells of a 48-hour-old culture are harvested by centrifugation (3500xg, 5 min) and washed in 2 ml of buffer I (100 mM potassium phosphate buffer, pH 7.0). The cell pellet is taken up in 500 µl of buffer 1 (cytosolic proteins) or 2 (100 mM potassium phosphate buffer pH7.0; 1% Triton X-100) (total proteins), and 1 µl of 500 mM PMSF in isopropanol is added. 500 µl of glass beads (d= 0.5 mm) are added to the cells, and the cells are disrupted by vortexing 5x for one minute. The liquid between the glass beads is transferred into a fresh Eppendorf tube. Cell debris and membrane components are removed by centrifuging for 15 minutes (14000xg). The supernatant is transferred into a fresh Eppendorf tube and constitutes the protein fraction.

The HMG-CoA activity is determined by measuring the consumption of $NADPH+H^+$ in the reduction of 3-hydroxy-3-methylglutaryl-CoA, which is added as a substrate.

In a reaction volume of 1000 µl there are added 20 µl of yeast protein isolate together with 910 µl of buffer I; 50 µl of 0.1 M DTT and 10 µl of 16 mM $NADPH+H^+$. The reaction mixture is warmed to 30°C and is measured in a photometer for 7.5 minutes at 340 nm. The decrease in NADPH which is measured during this period is the breakdown rate without added substrate and is taken into consideration as background.

Thereafter, substrate is added (10 µl of 30 mM HMG-CoA), and the measurement is continued for 7.5 minutes. The HMG-CoA reductase activity is calculated by determining the specific NADPH breakdown rate.

Lanosterol C14-demethylase activity is understood as meaning the enzyme activity of a lanosterol C14-demethylase.

A lanosterol C14-demethylase is understood as meaning a protein with the enzymatic activity of converting lanosterol into 4,4-dimethylcholesta-8,14,24-trienol.

Accordingly, lanosterol C14-demethylase activity is understood as meaning the amount of lanosterol converted, or the amount of 4,4-dimethylcholesta-8,14,24-trienol formed, by the protein lanosterol C14-demethylase within a specific period of time.

Thus, in the case of an increased lanosterol C14-demethylase activity in comparison with the wild type, the amount of lanosterol converted, or the amount of 4,4-dimethylcholesta-8,14,24-trienol formed, by the protein lanosterol C14-demethylase within a specific period of time is increased in comparison with the wild type.

Preferably, this increase in the lanosterol C14-demethylase activity amounts to at least 5%, more preferably to at least 20%, more preferably to at least 50%, more preferably to at least 100%, even more preferably to at least 300%, especially preferably to at least 500%, in particular to at least 600%, of the lanosterol C14-demethylase activity of the wild type.

The lanosterol C14-demethylase activity is determined as described in Omura, T and Sato, R. (1964) The carbon monoxide binding pigment in liver microsomes. J.Biol.Chem. 239, 2370-2378. In this test, the amount of P450 enzyme is semiquantifiable as the holoenzyme with bound heme. The (active) holoenzyme (with heme) can be reduced by CO, and only the CO-reduced enzyme has an absorption maximum at 450 nm. Thus, the absorption maximum at 450 nm is a measure for the lanosterol C14-demethylase activity.

To carry out the activity determination, a microsome fraction (4-10 mg/ml protein in 100 mM potassium phosphate buffer) is diluted 1:4 in such a way that the protein concentration employed for the assay is 2 mg/ml. The assay is carried out directly in a cell.

A spatula-tipful of dithionite ($S_2O_4Na_2$) is added to the microsomes. The baseline is recorded with a spectrophotometer in the 380-500 nm range.

Approximately 20-30 CO bubbles are subsequently bubbled through the sample. The absorption is now measured in the same range. The absorption level at 450 nm corresponds to the amount of P450 enzyme in the reaction mixture.

Squalene epoxidase activity is understood as meaning the enzyme activity of a squalene epoxidase.

A squalene epoxidase is understood as meaning a protein with the enzymatic activity of converting squalene into squalene epoxide.

Accordingly, squalene epoxidase activity is understood as meaning the amount of squalene converted, or the amount of squalene epoxide formed, by the protein squalene epoxidase within a specific period of time.

Thus, in the case of an increased squalene epoxidase activity in comparison with the wild type, the amount of squalene converted, or the amount of squalene epoxide formed, by the protein squalene epoxidase within a specific period of time is increased in comparison with the wild type.

Preferably, this increase in squalene epoxidase activity amounts to at least 5%, more preferably to at least 20%, more preferably to at least 50%, more preferably to at least 100%, even more preferably to at least 300%, especially preferably to at least 500%, in particular to at least 600% of the squalene epoxidase activity of the wild type.

The squalene epoxidase activity is determined as described in Leber R, Landl K, Zinser E, Ahorn H, Spok A, Kohlwein SD, Turnowsky F, Daum G. (1998) Dual localization of squalene epoxidase, Erg1p, in yeast reflects a relationship between the endoplasmic reticulum and lipid particles, Mol. Biol. Cell. 1998, Feb;9(2):375-86.

This method comprises 0.35 to 0.7 mg of microsomal protein or 3.5 to 75 µg of lipid particle protein in 100mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.1 mM FAD, 3 mM NADPH, 0.1 mM squalene 2,3-epoxidase cyclase inhibitor U18666A, 32 µM [^{3}H]squalene dispersed in 0.005% Tween 80 in a total volume of 500 µl.

The assay is carried out at 30°C. After pretreatment for 10 minutes, the reaction is started by addition of squalene and stopped after 15, 30 or 45 minutes by lipid extraction with 3 ml of chloroform/methanol (2:1 vol/vol) and 750 µl 0.035% $MgCl_2$.

The lipids are dried under nitrogen and redissolved in 0.5 ml of chloroform/methanol (2:1 vol/vol). For a thin-layer chromatography, portions are placed on a silica gel 60 plate (0.2 mm) and separated with chloroform as the eluant. The positions containing [^{3}H]2,3-oxidosqualene and [^{3}H]squalene were scraped out and quantified with a scintillation counter.

Squalene synthetase activity is understood as meaning the enzyme activity of a squalene synthetase.

Squalene synthetase is understood as meaning a protein with the enzymatic activity of converting farnesyl pyrophosphate into squalene.

Accordingly, squalene synthetase activity is understood as meaning the amount of farnesyl pyrophosphate converted, or the amount of squalene formed, by the protein squalene synthetase within a specific period of time.

Thus, in the case of an increased squalene synthetase activity in comparison with the wild type, the amount of farnesyl pyrophosphate converted, or the amount of squalene formed, by the protein squalene synthetase within a specific period of time is increased in comparison with the wild type.

Preferably, this increase in squalene synthetase activity amounts to at least 5%, more preferably to at least 20%, more preferably to at least 50%, more preferably to at least 100%, even more preferably to at least 300%, especially preferably to at least 500%, in particular to at least 600% of the squalene synthetase activity of the wild type.

The squalene synthetase activity can be determined as described hereinbelow:

The reaction mixtures comprise 50 mM Mops, pH 7.2, 10 mM $MgCl_2$, 1% (v/v) Tween-80, 10% (v/v) 2-propanol, 1 mM DTT, 1 mg/ml BSA, NADPH, FPP (or PSPP) and microsomes (protein content 3 mg) in a total volume of 200 µl in glass tubes. Reactions with radioactive substrate [1-^{3}H]FPP (15-30 mCi/µmol) are incubated for 30 minutes at 30°C, and the suspension mixture is filled up with 1 volume of 1:1 (v/v) 40% aqueous KOH:methanol. Liquid NaCl is added until the solution is saturated, and 2 ml of ligroin comprising 0.5% (v/v) squalene are likewise added.

The suspension is vortexed for 30 seconds. Using a Pasteur pipette, 1 ml portions of the ligroin layer are applied to a packed 0.5 × 6 cm aluminum column (80-200 mesh, Fisher). The column is preequilibrated with 2 ml of ligroin comprising 0.5% (v/v) squalene. The column is subsequently eluted with 5 × 1 ml toluene comprising 0.5% (v/v) squalene. The squalene radioactivity is measured in Cytoscint (ICN) scintillation cocktail using a scintillation counter (Beckman).

This method is a modification of the methods described in Radisky et al., Biochemistry. 2000 Feb 22;39(7):1748-60, Zhang et al. (1993) *Arch. Biochem. Biophys. 304*, 133-143 and Poulter, C. D. et al. (1989) *J. Am. Chem. Soc. 111*, 3734-3739.

A wild type is understood as meaning the corresponding non-genetically-modified starting organism. Preferably, and in particular in cases where the organism or the wild

type are not unambiguously identifiable, the wild type for the reduction of the Δ22-desaturase activity, the increase in the HMG-CoA reductase activity, the increase in the lanosterol C14-demethylase activity, the increase in the squalene epoxidase activity and the increase in the squalene synthetase activity, and for the increase in the content in ergosta-5,7-dienol and/or its biosynthetic intermediates and/or metabolites is understood as meaning a reference organism. This reference organism is preferably the yeast strain Saccharomyces cerevisiae AH22.

The HMG-CoA reductase activity, the lanosterol C14-demethylase activity, the squalene epoxidase activity or the squalene synthetase activity can be increased independently in various ways, for example by eliminating inhibiting regulatory mechamisms at the expression and protein level, or by increasing the gene expression of the corresponding nucleic acids, that is to say nucleic acids encoding an HMG-CoA reductase, lanosterol C14-demethylase, squalene epoxidase or squalene synthetase, in comparison with the wild type.

Increasing the gene expression of the corresponding nucleic acid in comparison with the wild type can likewise be effected in various ways, for example by inducing the corresponding genes by activators, that is to say by inducing the HMG-CoA reductase gene, the lanosterol C14-demethylase gene, the squalene epoxidase gene or the squalene synthetase gene by activators or by introducing one or more gene copies of the corresponding nucleic acids, that is to say by introducing, into the organism, one or more nucleic acids encoding an HMG-CoA reductase, lanosterol C14-demethylase, squalene epoxidase or squalene synthetase.

In accordance with the invention, increasing the gene expression of a nucleic acid encoding an HMG-CoA reductase, lanosterol C14-demethylase, squalene epoxidase or squalene synthetase is also understood as meaning the manipulation of the expression of the organism's own, in particular the yeast's own, endogenous HMG-CoA reductases, lanosterol C14-demethylases, squalene epoxidases or squalene synthetases.

This can be achieved for example by modifying the promoter DNA sequence for genes encoding HMG-CoA reductase, lanosterol C14-demethylase, squalene epoxidase or squalene synthetase. Such a modification, which results in an increased expression rate of the gene in question, can be brought about for example by deletion or insertion of DNA sequences.

As described above, it is possible to modify the expression of the endogenous HMG-CoA reductase, lanosterol C14-demethylase, squalene epoxidase or squalene synthetase by applying exogenous stimuli. This can be brought about by specific

physiological conditions, that is to say by the application of foreign substances.

Moreover, a modified or increased expression of endogenous HMG-CoA reductase, lanosterol C14-demethylase, squalene epoxidase or squalene synthetase genes can be achieved by a regulator protein which does not occur in the nontransformed organism interacts with the promoter of these genes.

Such a regulator may be a chimeric protein consisting of a DNA binding domain and a transcription activator domain, as described, for example, in WO 96/06166.

In a preferred embodiment, the increase in lanosterol C14-demethylase activity in comparison with the wild type is effected by increasing the gene expression of a nucleic acid encoding a lanosterol C14-demethylase.

In a furthermore preferred embodiment, the increase in the gene expression of a nucleic acid encoding a lanosterol C14-demethylase is effected by introducing, into the organism, one or more nucleic acids encoding a lanosterol C14-demethylase.

In principle, any lanosterol C14-demethylase gene (ERG11), that is to say any nucleic acid encoding a lanosterol C14-demethylase, may be used for this purpose. In the case of genomic lanosterol C14-demethylase nucleic acid sequences from eukaryotic sources, which contain introns, and in the event that the host organism is not capable, or cannot be made capable, of expressing the corresponding lanosterol C14-demethylase, it is preferred to use preprocessed nucleic acid sequences, such as the corresponding cDNAs.

Examples of lanosterol C14-demethylase genes are nucleic acids encoding a lanosterol C14-demethylase from *Saccharomyces cerevisiae* (Kalb VF, Loper JC, Dey CR, Woods CW, Sutter TR (1986) Isolation of a cytochrome P-450 structural gene from *Saccharomyces cerevisiae*. Gene 45(3):237-45), *Candida albicans* (Lamb DC , Kelly DE, Baldwin BC, Gozzo F, Boscott P, Richards WG, Kelly SL (1997) Differential inhibition of *Candida albicans* CYP51 with azole antifungal stereoisomers. FEMS Microbiol Lett 149(1):25-30), *Homo sapiens* (Stromstedt M, Rozman D, Waterman MR. (1996) The ubiquitously expressed human CYP51 encodes lanosterol 14 alpha-demethylase, a cytochrome P450 whose expression is regulated by oxysterols. Arch Biochem Biophys 1996 May 1;329(1):73-81c) or *Rattus norvegicus*, Aoyama Y, Funae Y, Noshiro M, Horiuchi T, Yoshida Y. (1994) Occurrence of a P450 showing high homology to yeast lanosterol 14-demethylase (P450(14DM)) in the rat liver. Biochem Biophys Res Commun. Jun 30;201(3):1320-6).

In the transgenic organisms according to the invention, there thus exists, in this preferred embodiment, at least one further lanosterol C14-demethylase gene in comparison with the wild type.

The number of the lanosterol C14-demethylase genes in the transgenic organisms according to the invention is at least two, preferably more than two, especially preferably more than three, very especially preferably more than five.

All of the nucleic acids mentioned in the description may be, for example, an RNA, DNA or cDNA sequence.

The above-described method preferably employs nucleic acids encoding proteins comprising the amino acid sequence SEQ. ID. NO. 6 or a sequence derived from this sequence by substitution, insertion or deletion of amino acids which has at least 30%, preferably at least 50%, more preferably at least 70%, especially preferably at least 90%, most preferably at least 95% identity with the sequence SEQ. ID. NO. 6 at the amino acid level, which proteins have the enzymatic characteristic of a lanosterol C14-demethylase.

The sequence SEQ. ID. NO. 6 constitutes the amino acid sequence of the *Saccharomyces cerevisiae* lanosterol C14-demethylase.

Further examples of lanosterol C14-demethylases and lanosterol C14-demethylase genes can be found readily, for example from various organisms whose genomic sequence is known, by homology comparisons of the amino acid sequences or of the corresponding backtranslated nucleic acid sequences from databases with SEQ. ID. NO. 2.

Further examples of lanosterol C14-demethylases and lanosterol C14-demethylase genes can be found readily in a manner known per se by hybridization and PCR techniques from various organisms whose genomic sequence is not known, for example starting from the sequence SEQ. ID. NO. 5.

In the description, the term "substitution" is understood as meaning the substitution of one or more amino acids by one or more amino acids. It is preferred to perform what are known as conservative substitutions, in which the replacement amino acid has a similar property to the original amino acid, for example the substitution of Glu by Asp, Gln by Asn, Val by Ile, Leu by Ile, Ser by Thr.

Deletion is the replacement of an amino acid by a direct bond. Preferred positions for deletions are the termini of the polypeptide and the linkages between the individual protein domains.

Insertions are introductions of amino acids into the polypeptide chain, a direct bond formally being replaced by one or more amino acids.

Identity between two proteins is understood as meaning the identity of the amino acids over in each case the entire protein length, in particular the identity calculated by alignment with the aid of the Lasergene software from DNASTAR, inc.Madison, Wisconsin (USA) using the Clustal method (Higgins DG, Sharp PM. Fast and sensitive multiple sequence alignments on a microcomputer. Comput Appl. Biosci. 1989 Apr;5(2):151-1), setting the following parameters:

Multiple alignment parameter:
Gap penalty 10
Gap length penalty 10
Pairwise alignment parameter:
K-tuple 1
Gap penalty 3
Window 5
Diagonals saved 5

Accordingly, a protein with an identity of at least 30% with the sequence SEQ. ID. NO. 6 at the amino acid level is understood as meaning a protein which has at least 30% identity when its sequence is aligned with the sequence SEQ. ID. NO. 6, in particular in accordance with the above program algorithm with the above parameter set.

In a furthermore preferred embodiment, nucleic acids encoding proteins comprising the amino acid sequence of the *Saccharomyces cerevisiae* lanosterol C14-demethylase (SEQ. ID. NO. 6) are introduced into organisms.

Suitable nucleic acid sequences can be obtained for example by backtranslating the polypeptide sequence in accordance with the genetic code.

Codons which are preferably used for this purpose are those which are used frequently in accordance with the organism-specific codon usage. The codon usage can be determined readily with the aid of computer evaluations of other, known genes of the

organisms in question.

If, for example, the protein is to be expressed in yeast, it is frequently advantageous to use the yeast codon usage when backtranslating.

In an especially preferred embodiment, a nucleic acid comprising the sequence SEQ. ID. NO. 5 is introduced into the organism.

The sequence SEQ. ID. NO. 5 constitutes the genomic DNA from *Saccharomyces cerevisiae* (ORF S0001049), which encodes the lanosterol C14-demethylase with the sequence SEQ ID NO. 6.

All of the abovementioned lanosterol C14-demethylase genes can furthermore be generated from the nucleotide units by chemical synthesis in a manner known per se, such as, for example, by fragment condensation of individual overlapping complementary nucleic acid units of the double helix. Oligonucleotides can be synthesized chemically for example in a known manner using the phosphoamidite method (Voet, Voet, 2nd Edition, Wiley Press New York, pages 896-897). The annealment of synthetic oligonucleotides and the filling in of gaps with the aid of the DNA polymerase Klenow fragment and ligation reactions are described in Sambrook et al. (1989), Molecular cloning: A laboratory manual, Cold Spring Harbor Laboratory Press, as are general cloning methods.

In a preferred embodiment, increasing the HMG-CoA reductase activity in comparison with the wild type is effected by increasing the gene expression of a nucleic acid encoding an HMG-CoA reductase.

In an especially preferred embodiment of the method according to the invention, increasing the gene expression of a nucleic acid encoding an HMG-CoA reductase is effected by introducing, into the organism, a nucleic acid construct comprising a nucleic acid encoding an HMG-CoA reductase whose expression in the organism is subject to reduced regulation in comparison with the wild type.

Reduced regulation in comparison with the wild type is understood as meaning a regulation which is reduced in comparison with the above-defined wild type, preferably no regulation, at the expression or protein level.

The reduced regulation can preferably be achieved by means of a promoter which is operably linked to the coding sequence in the nucleic acid construct and which, in the

organism, is subject to reduced regulation in comparison with the wild-type promoter.

For example, the middle ADH promoter in yeast is only subject to reduced regulation and is therefore particularly preferred as promoter in the above-described nucleic acid construct.

This promoter fragment of the *ADH1*2s promoter, hereinbelow also referred to as *ADH1*, shows almost constitutive expression (Ruohonen L, Penttila M, Keranen S. (1991) Optimization of Bacillus alpha-amylase production by *Saccharomyces cerevisiae*. Yeast. May-Jun;7(4):337-462; Lang C, Looman AC. (1995) Efficient expression and secretion of Aspergillus niger RH5344 polygalacturonase in *Saccharomyces cerevisiae*. Appl Microbiol Biotechnol. Dec;44(1-2):147-56.), so that the transcriptional regulation no longer proceeds via ergosterol biosynthesis intermediates.

Further preferred promoters with reduced regulation are constitutive promoters such as, for example, the yeast TEF1 promoter, the yeast GPD promoter or the yeast PGK promoter (Mumberg D, Muller R, Funk M.(1995) Yeast vectors for the controlled expression of heterologous proteins in different genetic backgrounds. Gene. 1995 Apr 14;156(1):119-22; Chen CY, Oppermann H, Hitzeman RA.(1984) Homologous versus heterologous gene expression in the yeast, *Saccharomyces cerevisiae*. Nucleic Acids Res. Dec 11;12(23):8951-70.).

In a further preferred embodiment, reduced regulation can be achieved by using, as the nucleic acid encoding an HMG-CoA reductase, a nucleic acid whose expression in the organism is subject to reduced regulation in comparison with the homologous, orthologous nucleic acid.

It is especially preferred to use a nucleic acid which only encodes the catalytic region of the HMG-CoA reductase (truncated (t-)HMG-CoA reductase) as the nucleic acid encoding an HMG-CoA reductase. This nucleic acid (t-HMG), which is described in EP 486 290 and WO 99/16886, only encodes the catalytically active portion of the HMG-CoA reductase while the membrane domain, which is responsible for the regulation at the protein level, is absent. Thus, this nucleic acid is subjected to reduced regulation, in particular in yeast, and leads to an increased gene expression of the HMG-CoA reductase.

The above-described nucleic acid construct can be incorporated into the host organism either chromosomally using integration vectors or episomally using episomal plasmids, in each case comprising the above-described nucleic acid construct.

In an especially preferred embodiment, nucleic acids are introduced, preferably via the above-described nucleic acid construct, which encode proteins comprising the amino acid sequence SEQ. ID. NO. 4 or a sequence derived from this sequence by substitution, insertion or deletion of amino acids which has at least 30% identity with the sequence SEQ. ID. NO. 4 at the amino acid level, which proteins have the enzymatic characteristic of an HMG-CoA reductase.

The sequence SEQ. ID. NO. 4 constitutes the amino acid sequence of the truncated HMG-CoA reductase (t-HMG).

Further examples of HMG-CoA reductases, and thus also of the t-HMG-CoA reductases which are reduced to the catalytic portion, or the coding genes, can be found readily, for example from various organisms whose genomic sequence is known, by homology comparisons of the amino acid sequences or of the corresponding back-translated nucleic acid sequences from databases with SEQ ID. NO. 4.

Further examples of HMG-CoA reductases, and thus also of the t-HMG-CoA reductases which are reduced to the catalytic portion, or the coding genes, can be found readily from various organisms whose genomic sequence is not known by hybridization and PCR techniques in a manner known per se, for example starting from the sequence SEQ. ID. No. 3.

It is especially preferred to use a nucleic acid comprising the sequence SEQ. ID. NO. 3 as nucleic acid encoding a truncated HMG-CoA reductase.

In an especially preferred embodiment, the reduced regulation is achieved by using, as nucleic acid encoding an HMG-CoA reductase, a nucleic acid whose expression in the organism is subject to reduced regulation in comparison with the organism's own, orthologous nucleic acid and by using a promoter which is subject to reduced regulation in the organism in comparison with the wild-type promoter.

In a preferred embodiment, increasing the squalene epoxidase activity in comparison with the wild type is effected by increasing the gene expression of a nucleic acid encoding a squalene epoxidase.

In a furthermore preferred embodiment, increasing the gene expression of a nucleic acid encoding a squalene epoxidase is effected by introducing, into the organism, one or more nucleic acids encoding squalene epoxidase.

In principle, any squalene epoxidase gene (ERG1), that is to say any nucleic acid which encodes a squalene epoxidase, may be used for this purpose. In the case of genomic squalene epoxidase nucleic acid sequences from eukaryotic sources, which contain introns, and in the event that the host organism is not capable, or cannot be made capable, of expressing the corresponding squalene epoxidase, it is preferred to use preprocessed nucleic acid sequences, such as the corresponding cDNAs.

Examples of nucleic acids encoding a squalene epoxidase are nucleic acids encoding a squalene epoxidase from *Saccharomyces cerevisiae* (Jandrositz, A., et al (1991) The gene encoding squalene epoxidase from *Saccharomyces cerevisiae:* cloning and characterization. Gene 107:155-160, from *Mus musculus* (Kosuga K, Hata S, Osumi T, Sakakibara J, Ono T. (1995) Nucleotide sequence of a cDNA for mouse squalene epoxidase, Biochim Biophys Acta, Feb 21;1260(3):345-8b), from Rattus norvegicus (Sakakibara J, Watanabe R, Kanai Y, Ono T. (1995) Molecular cloning and expression of rat squalene epoxidase. J Biol Chem Jan 6;270(1):17-20c) or from *Homo sapiens* (Nakamura Y, Sakakibara J, Izumi T, Shibata A, Ono T. (1996) Transcriptional regulation of squalene epoxidase by sterols and inhibitors in HeLa cells., J. Biol. Chem. 1996, Apr 5;271(14):8053-6).

In the transgenic organisms according to the invention, there thus exists, in this preferred embodiment, at least one further squalene epoxidase gene in comparison with the wild type.

The number of the squalene epoxidase genes in the transgenic organisms according to the invention is at least two, preferably more than two, especially preferably more than three, very especially preferably more than five.

The above-described method preferably employs nucleic acids encoding proteins comprising the amino acid sequence SEQ. ID. NO. 8 or a sequence derived from this sequence by substitution, insertion or deletion of amino acids which has at least 30%, preferably at least 50%, more preferably at least 70%, especially preferably at least 90%, most preferably at least 95% identity with the sequence SEQ. ID. NO. 8 at the amino acid level, which proteins have the enzymatic characteristic of a squalene epoxidase.

The sequence SEQ. ID. NO. 8 constitutes the amino acid sequence of the *Saccharomyces cerevisiae* squalene epoxidase.

Further examples of squalene epoxidases and squalene epoxidase genes can be found readily, for example from various organisms whose genomic sequence is known,

by homology comparisons of the amino acid sequences or of the corresponding backtranslated nucleic acid sequences from databases with SEQ. ID. NO. 8.

Further examples of squalene epoxidase and squalene epoxidase genes can be found readily in a manner known per se by hybridization and PCR techniques from various organisms whose genomic sequence is not known, for example starting from the sequence SEQ. ID. NO. 7.

In a furthermore preferred embodiment, nucleic acids encoding proteins comprising the amino acid sequence of the *Saccharomyces cerevisiae* squalene epoxidase (SEQ. ID. NO. 8) are introduced into organisms.

Suitable nucleic acid sequences can be obtained for example by backtranslating the polypeptide sequence in accordance with the genetic code.

Codons which are preferably used for this purpose are those which are used frequently in accordance with the organism-specific codon usage. The codon usage can be determined readily with the aid of computer evaluations of other, known genes of the organisms in question.

If, for example, the protein is to be expressed in yeast, it is frequently advantageous to use the yeast codon usage when backtranslating.

In an especially preferred embodiment, a nucleic acid comprising the sequence SEQ. ID. NO. 7 is introduced into the organism.

The sequence SEQ. ID. NO. 7 constitutes the genomic DNA from *Saccharomyces cerevisiae* (ORF S0003407), which encodes the squalene epoxidase with the sequence SEQ ID NO. 8.

All of the abovementioned squalene epoxidase genes can furthermore be generated from the nucleotide units by chemical synthesis in a manner known per se, such as, for example, by fragment condensation of individual overlapping complementary nucleic acid units of the double helix. Oligonucleotides can be synthesized chemically for example in a known manner using the phosphoamidite method (Voet, Voet, 2nd Edition, Wiley Press New York, pages 896-897). The annealment of synthetic oligonucleotides and the filling in of gaps with the aid of the DNA polymerase Klenow fragment and ligation reactions are described in Sambrook et al. (1989), Molecular cloning: A laboratory manual, Cold Spring Harbor Laboratory Press, as are general cloning

methods.

In a preferred embodiment, increasing the squalene synthetase activity in comparison with the wild type is effected by increasing the gene expression of a nucleic acid encoding a squalene synthetase.

In a furthermore preferred embodiment, increasing the gene expression of a nucleic acid encoding a squalene synthetase is effected by introducing, into the organism, one or more nucleic acids encoding a squalene synthetase.

In principle, any squalene synthetase gene (ERG9), that is to say any nucleic acid which encodes a squalene synthetase, may be used for this purpose. In the case of genomic squalene synthetase nucleic acid sequences from eukaryotic sources, which contain introns, and in the event that the host organism is not capable, or cannot be made capable, of expressing the corresponding squalene synthetase, it is preferred to use preprocessed nucleic acid sequences, such as the corresponding cDNAs.

Examples of nucleic acids encoding a squalene synthetase are nucleic acids encoding a squalene synthetase from *Saccharomyces cerevisiae (ERG9)*, (Jennings, S.M., (1991): Molecular cloning and characterization of the yeast gene for squalene synthetase. Proc Natl Acad Sci USA. Jul15;88(14):6038-42), nucleic acids encoding a squalene synthetase from *Botryococcus braunii Okada* (Devarenne, T.P. et al.: Molecular characterization of squalene synthase from the green microalga Botryococcus braunii, raceB, Arch. Biochem. Biophys. 2000, Jan15, 373(2):307-17), nucleic acids encoding a squalene synthetase from potato tuber (Yoshioka H. et al.: cDNA cloning of sesquiter penecyclase and squalene synthase, and expression of the genes in potato tuber infected with Phytophthora infestans, Plant. Cell. Physiol.1999, Sep;40(9):993-8) or nucleic acids encoding a squalene synthetase from *Glycyrrhiza glabra* (Hayashi, H. et al.: Molecular cloning and characterization of two cDNAs for Glycyrrhiza glabra squalene synthase, Biol. Pharm. Bull. 1999, Sep;22(9):947-50.

In the transgenic organisms according to the invention, there thus exists, in this preferred embodiment, at least one further squalene synthetase gene in comparison with the wild type.

The number of the squalene synthetase genes in the transgenic organisms according to the invention is at least two, preferably more than two, especially preferably more than three, very especially preferably more than five.

The above-described method preferably employs nucleic acids encoding proteins comprising the amino acid sequence SEQ. ID. NO. 10 or a sequence derived from this sequence by substitution, insertion or deletion of amino acids which has at least 30%, preferably at least 50%, more preferably at least 70%, especially preferably at least 90%, most preferably at least 95% identity with the sequence SEQ. ID. NO. 10 at the amino acid level, which proteins have the enzymatic characteristic of a squalene synthetase.

The sequence SEQ. ID. NO. 10 constitutes the amino acid sequence of the *Saccharomyces cerevisiae* squalene synthetase (ERG9).

Further examples of squalene synthetases and squalene synthetase genes can be found readily, for example from various organisms whose genomic sequence is known, by homology comparisons of the amino acid sequences or of the corresponding backtranslated nucleic acid sequences from databases with SEQ. ID. NO. 10.

Further examples of squalene synthetases and squalene synthetase genes can be found readily in a manner known per se by hybridization and PCR techniques from various organisms whose genomic sequence is not known, for example starting from the sequence SEQ. ID. NO. 9.

In a furthermore preferred embodiment, nucleic acids encoding proteins comprising the amino acid sequence of the *Saccharomyces cerevisiae* squalene synthetase (SEQ. ID. NO. 10) are introduced into organisms.

Suitable nucleic acid sequences can be obtained for example by backtranslating the polypeptide sequence in accordance with the genetic code.

Codons which are preferably used for this prupose are those which are used frequently in accordance with the organism-specific codon usage. The codon usage can be determined readily with the aid of computer evaluations of other, known genes of the organisms in question.

If, for example, the protein is to be expressed in yeast, it is frequently advantageous to use the codon usage of yeast when backtranslating.

In an especially preferred embodiment, a nucleic acid comprising the sequence SEQ. ID. NO. 9 is introduced into the organism.

The sequence SEQ. ID. NO. 9 constitutes the genomic DNA from *Saccharomyces cerevisiae* (ORF YHR190W), which encodes the squalene synthetase of the sequence SEQ. ID. NO. 10.

All of the abovementioned squalene synthetase genes can furthermore be generated from the nucleotide units by chemical synthesis in a manner known per se, such as, for example, by fragment condensation of individual overlapping complementary nucleic acid units of the double helix. Oligonucleotides can be synthesized chemically for example in a known manner using the phosphoamidite method (Voet, Voet, 2nd Edition, Wiley Press New York, pages 896-897). The annealment of synthetic oligonucleotides and the filling in of gaps with the aid of the DNA polymerase Klenow fragment and ligation reactions are described in Sambrook et al. (1989), Molecular cloning: A laboratory manual, Cold Spring Harbor Laboratory Press, as are general cloning methods.

The organisms cultured in the method according to the invention are organisms which have a reduced Δ22-desaturase activity and an increased HMG-CoA reductase activity and an increased activity of at least one of the activities selected from the group consisting of lanosterol C14-demethylase activity, squalene epoxidase activity and squalene synthetase activity in comparison with the wild type.

In a preferred embodiment, the organisms cultured are organisms which have a reduced Δ22-desaturase activity and an increased HMG-CoA reductase activity and an increased lanosterol C14-demethylase activity, squalene epoxidase activity or squalene synthetase activity in comparison with the wild type.

In an especially preferred embodiment of the method according to the invention, the organisms have a reduced Δ22-desaturase activity and an increased HMG-CoA reductase activity and an increased activity of at least two of the activities selected from the group consisting of lanosterol C14-demethylase activity, squalene epoxidase activity and squalene synthetase activity in comparison with the wild type.

Especially preferred combinations are a reduced Δ22-desaturase activity and an increased HMG-CoA reductase activity and an increased lanosterol C14-demethylase activity and squalene epoxidase activity or lanosterol C14-demethylase activity and squalene synthetase activity or an increased squalene epoxidase activity and squalene synthetase activity in comparison with the wild type.

In a very especially preferred embodiment of the method according to the invention, the organisms have a reduced Δ22-desaturase activity and an increased HMG-CoA

reductase activity and an increased lanosterol C14-demethylase activity and an increased squalene epoxidase activity and an increased squalene synthetase activity in comparison with the wild type.

Organisms or genetically modified organisms are understood as meaning, in accordance with the invention, for example bacteria, in particular bacteria of the genus *Bacillus, Escherichia coli, Lactobacillus spec.* or *Streptomyces spec.*,

for example yeasts, in particular yeasts of the genus *Saccharomyces cerevisiae, Pichia pastoris* or *Klyveromyces spec.*,

for example fungi, in particular fungi of the genus *Aspergillus spec., Penicillium spec.* or *Dictyostelium spec.*,

and, for example, also insect cell lines which are capable of generating ergosta-5,7-dienol and/or its biosynthetic intermediates and/or metabolites, either as the wild type or owing to preceding genetic modification.

Especially preferred organisms or genetically modified organisms are yeasts, in particular of the species *Saccharomyces cerevisiae*, in particular the yeast strains *Saccharomyces cerevisiae AH22, Saccharomyces cerevisiae* GRF, *Saccharomyces cerevisiae* DBY747 and *Saccharomyces cerevisiae* BY4741.

The biosynthetic intermediates of ergosta-5,7-dienol are understood as meaning all those compounds which occur as intermediates in the ergosta-5,7-dienol biosynthesis in the organism used, preferably the compounds mevalonate, farnesyl pyrophosphate, geraniol pyrophosphate, squalene epoxide, 4-dimethylcholesta-8,14,24-trienol, 4,4-dimethylzymosterol, squalene, farnesol, geraniol, lanosterol, zymosterone and zymosterol.

The biosynthetic metabolites of ergosta-5,7-dienol are understood as meaning all those compounds which are biosynthetic derivatives of ergosta-5,7-dienol in the organism used, that is to say in which ergosta-5,7-dienol occurs as intermediate. They may be compounds which the organism used produces naturally from ergosta-5,7-dienol.

However, they are also understood as meaning compounds which can only be produced from ergosta-5,7-dienol in the organism by introducing genes and enzyme activities from other organisms to which the starting organism has no orthologous gene.

Owing to the introduction of further plant genes and/or mammalian genes into yeast it is possible, for example, to produce biosynthetic ergosta-5,7-dienol metabolites which only occur naturally in plants and/or mammals in this yeast.

The introduction into yeast of, for example, nucleic acids encoding a plant Δ7-reductase (DWF5) or its functional equivalents or variants and of nucleic acids encoding mature forms of CYP11A1, ADX(FDX1), ADR (FDXR) and 3β-HSD or their functional equivalents or variants leads to the biosynthesis of progesterone in this yeast. A detailed description of the procedure and of the methods and materials for the corresponding genetic modification of yeast is published in C. Duport et al., Nat. Biotechnol. 1998, 16, 186-189 and in the references cited therein, which are herewith expressly incorporated by reference.

The introduction into yeast of, for example, nucleic acids encoding a plant Δ7-reductase (DWF5) or its functional equivalents or variants and of nucleic acids encoding mature forms of CYP11A1, ADX(FDX1) and ADR (FDXR) or their functional equivalents or variants and of nucleic acids encoding mitochondrial forms of ADX and CYP11B1, 3b-HSD, CYP17A1 and CYP21A1 or their functional equivalents or variants leads to the biosynthesis of hydrocortisone, 11-deoxycortisol, corticosterone and acetalpregnenolone.

To further increase the content in biosynthetic ergosta-5,7-dienol metabolites such as, for example, hydrocortisone, it is additionally advantageous to suppress wasteful metabolic pathways, that is to say biosynthetic pathways which do not lead to the desired product. For example, the reduction of the activities of the gene products of ATF2, GCY1 and YPR1, especially preferably the deletion of these activities, in yeast leads to a further increase in the hydrocortisone content.

A detailed description of this procedure and of the methods and materials for the corresponding genetic modification of yeast is published in F.M. Szczebara et al., Nat. Biotechnol. 2003, 21, 143-149 and in the references cited therein, which are herewith expressly incorporated by reference.

The biosynthetic ergosta-5,7-dienol metabolites are therefore understood as meaning in particular campesterol, pregnenolone, 17-OH pregnenolone, progesterone, 17-OH-progesterone, 11-deoxycortisol, hydrocortisone, deoxycorticosterone and/or corticosterone.

Preferred biosynthetic metabolites are progesterone, corticosterone and hydrocortisone, especially preferably hydrocortisone.

Some of the compounds produced in the method according to the invention are themselves steroid hormones and can be used for therapeutical purposes.

The compounds produced, such as, for example, ergosta-5,7-dienol or hydrocortisone, can furthermore be used for preparing steroid hormones or for the synthesis of active ingredients with a potent antiinflammatory, abortive or antiproliferative activity via biotransformation, chemical synthesis or biotechnological production.

In the method according to the invention for the production of ergosta-5,7-dienol and/or its biosynthetic intermediates and/or metabolites the step of culturing the genetically modified organisms, hereinbelow also referred to as transgenic organisms, is preferably followed by harvesting of the organisms and isolation of ergosta-5,7-dienol and/or its biosynthetic intermediates and/or metabolites from the organisms.

The organisms are harvested in a manner known per se to suit the organism in question. Microorganisms such as bacteria, mosses, yeasts and fungi or plant cells which are grown in liquid nutrient media by fermentation can be separated for example by centrifugation, decanting or filtration.

Ergosta-5,7-dienol and/or its biosynthetic intermediates and/or metabolites from the harvested biomass are isolated jointly or separately for each compound in a manner known per se, for example by extraction and, if appropriate, further chemical or physical purification processes such as, for example, precipitation methods, crystallography, thermal separation methods like rectification methods or physical separation methods such as, for example, chromatography.

The invention furthermore relates to a method for generating a genetically modified organism in which, starting from a starting organism, the Δ22-desaturase activity is reduced and the HMG-CoA reductase activity is increased and at least one of the activities selected from the group consisting of lanosterol C14-demethylase activity, squalene epoxidase activity and squalene synthetase activity is increased.

The methods for deleting the target locus Δ22-desaturase gene have already been detailed above.

The transgenic organisms, in particular yeasts, can preferably be generated by transforming the starting organisms, in particular yeasts, with a nucleic acid construct

comprising at least one nucleic acid encoding an HMG-CoA reductase and comprising at least one nucleic acid selected from the group consisting of nucleic acids encoding a lanosterol C14-demethylase, nucleic acids encoding a squalene epoxidase and nucleic acids encoding a squalene synthetase, which nucleic acids are linked operably to one or more regulatory signals which ensure the transcription and translation in the organisms. In this embodiment, the transgenic organisms are generated using a nucleic acid construct.

Nucleic acid constructs which can be used for this purpose are those which, in addition to the expression cassettes described hereinbelow and comprising promoter, coding sequence and, if appropriate, terminator, and in addition to a selection marker described hereinbelow, comprise, at their 3' and 5' ends, nucleic acid sequences which are identical to nucleic acid sequences at the beginning and at the end of the gene to be deleted.

However, the transgenic organisms may also preferably be generated by transforming the starting organisms, in particular yeasts, with a combination of nucleic acid constructs comprising nucleic acid constructs comprising at least one nucleic acid encoding an HMG-CoA reductase and comprising nucleic acid constructs or a combination of nucleic acid constructs comprising at least one nucleic acid selected from the group consisting of nucleic acids encoding a lanosterol C14-demethylase, nucleic acids encoding a squalene epoxidase and nucleic acids encoding a squalene synthetase and which are in each case linked operably to one or more regulatory signals which ensure the transcription and translation in organisms.
In this embodiment, the transgenic organisms are generated using individual nucleic acid constructs or a combination of nucleic acid constructs.

Nucleic acid constructs in which the coding nucleic acid sequence is linked operably to one or more regulatory signals which ensure the transcription and translation in organisms, in particular in yeasts, are hereinbelow also referred to as expression cassettes.

Nucleic acid constructs comprising this expression cassette are, for example, vectors or plasmids.

The regulatory signals preferably comprise one or more promoters which ensure the transcription and translation in organisms, in particular in yeasts.

The expression cassettes comprise regulatory signals, that is to say regulatory nucleic acid sequences which control the expression of the coding sequence in the host cell. In

accordance with a preferred embodiment, an expression cassette encompasses a promoter upstream, i.e. at the 5' end of the coding sequence, and a terminator downstream, i.e. at the 3' end, and, if appropriate, further regulatory elements which are linked operably to the interposed coding sequence for at least one of the above-described genes.

Operable linkage is understood as meaning the sequential arrangement of promoter, coding sequence, if appropriate terminator and if appropriate further regulatory elements in such a way that each of the regulatory elements can fulfill its intended function upon expression of the coding sequence.

By way of example, the preferred nucleic acid constructs, expression cassettes and plasmids for yeasts and fungi and methods for generating transgenic yeasts and the transgenic yeasts themselves are described in the following text.

A suitable promoter for the expression cassette is, in principle, any promoter which is capable of controlling the expression of foreign genes in organisms, in particular in yeasts.

A promoter which is preferably used is, in particular, a promoter which is subject to reduced regulation in yeast, such as, for example, the middle ADH promoter.

This promoter fragment of the *ADH12*s promoter, hereinbelow also referred to as *ADH1,* shows approximately constitutive expression (Ruohonen L, Penttila M, Keranen S. (1991) Optimization of Bacillus alpha-amylase production by *Saccharomyces cerevisiae*. Yeast. May-Jun;7(4):337-462; Lang C, Looman AC. (1995) Efficient expression and secretion of Aspergillus niger RH5344 polygalacturonase in *Saccharomyces cerevisiae*. Appl Microbiol Biotechnol. Dec;44(1-2):147-56.), so that transcriptional regulation is no longer effected by ergosterol biosynthesis intermediates.

Further preferred promoters with reduced regulation are constitutive promoters such as, for example, the yeast TEF1 promoter, the yeast GPD promoter or the yeast PGK promoter (Mumberg D, Muller R, Funk M.(1995) Yeast vectors for the controlled expression of heterologous proteins in different genetic backgrounds. Gene. 1995 Apr 14;156(1):119-22; Chen CY, Oppermann H, Hitzeman RA.(1984) Homologous versus heterologous gene expression in the yeast, *Saccharomyces cerevisiae*. Nucleic Acids Res. Dec 11;12(23):8951-70.).

The expression cassette may also comprise inducible promoters, in particular chemically inducible promoters, by means of which the expression, in the organism, of the

nucleic acids encoding an HMG-CoA reductase, lanosterol C14-demethylase, squalene epoxidase or squalene synthetase can be controlled at a particular point in time.

Such promoters such as, for example, the yeast Cupl promoter, (Etcheverry T. (1990) Induced expression using yeast copper metallothionein promoter. Methods Enzymol. 1990;185:319-29.), the yeast Gal1-10 promoter (Ronicke V, Graulich W, Mumberg D, Muller R, Funk M. (1997) Use of conditional promoters for expression of heterologous proteins in *Saccharomyces cerevisiae,* Methods Enzymol.283:313-22) or the yeast Pho5 promoter (Bajwa W, Rudolph H, Hinnen A.(1987) PHO5 upstream sequences confer phosphate control on the constitutive PHO3 gene. Yeast. 1987 Mar;3(1):33-42) may be used by way of example.

A suitable terminator for the expression cassette is, in principle, any terminator which is capable of controlling the expression of foreign genes in organisms, in particular in yeasts.

The yeast tryptophan terminator (TRP1 terminator) is preferred.

An expression cassette is preferably generated by fusing a suitable promoter to the above-described nucleic acids encoding an HMG-CoA reductase, lanosterol C14-demethylase, squalene epoxidase or squalene synthetase and, if appropriate, a terminator using customary recombination and cloning techniques as are described, for example, in T. Maniatis, E.F. Fritsch and J. Sambrook, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1989), in T.J. Silhavy, M.L. Berman and L.W. Enquist, Experiments with Gene Fusions, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1984) and in Ausubel, F.M. et al., Current Protocols in Molecular Biology, Greene Publishing Assoc. and Wiley-Interscience (1987).

The nucleic acids according to the invention can have been synthesized or obtained naturally or comprise a mixture of synthetic and natural nucleic acid components, or else consist of various heterologous gene segments from various organisms.

Preferred are, as described above, synthetic nucleotide sequences with codons which are preferred by yeasts. These codons which are preferred by yeasts can be determined from codons with the highest protein frequency which are expressed in most of the yeast species of interest.

When preparing an expression cassette, various DNA fragments can be manipulated in order to obtain a nucleotide sequence which expediently reads in the correct direction

and is equipped with the correct reading frame. Adapters or linkers may be added to the fragments in order to link the DNA fragments with one another.

The promoter and terminator regions may expediently be provided, in the direction of transcription, with a linker or polylinker comprising one or more restriction cleavage sites for the insertion of this sequence. As a rule, the linker has 1 to 10, in most cases 1 to 8, preferably 2 to 6, restriction cleavage sites. In general, the linker within the regulatory regions has a size of less than 100 bp, frequently less than 60 bp, but at least 5 bp. The promoter may be either native, or homologous, or else foreign, or heterologous, with respect to the host organism. The expression cassette preferably comprises, in the 5'-3' direction of transcription, the promoter, a coding nucleic acid sequence or a nucleic acid construct and a region for transcriptional termination. Various termination regions can be exchanged for one another as desired.

Manipulations which provide suitable restriction cleavage sites or which remove superfluous DNA or restriction cleavage sites may furthermore be employed. Where insertions, deletions or substitutions such as, for example, transitions and transversions, are suitable, *in vitro* mutagenesis, primer repair, restriction or ligation may be used.

Suitable manipulations such as, for example, restriction, chewing back or filling in overhangs for blunt ends may provide complementary ends of the fragments for the ligation.

The invention furthermore relates to the use of the above-described nucleic acids, the above-described nucleic acid constructs or the above-described proteins for the generation of transgenic organisms, in particular yeasts.

These transgenic organisms, in particular yeasts, preferably have an increased content in ergosta-5,7-dienol and/or its biosynthetic intermediates and/or metabolites in comparison with the wild type.

The invention furthermore relates to the use of the above-described nucleic acids or of the nucleic acid constructs according to the invention for increasing the content in ergosta-5,7-dienol and/or its biosynthetic intermediates and/or metabolites in organisms.

The above-described proteins and nucleic acids can be used for producing ergosta-5,7-dienol and/or its biosynthetic intermediates and/or metabolites in transgenic

organisms.

The transfer of foreign genes into the genome of an organism, in particular of yeast, is referred to as transformation.

Transformation methods which are known per se may be used for this purpose, in particular in yeasts.

Suitable methods for transforming yeasts are, for example, the LiAC method as described in Schiestl RH, Gietz RD. (1989) High efficiency transformation of intact yeast cells using single stranded nucleic acids as a carrier, Curr Genet. Dec;16(5-6):339-46, the electroporation as described in Manivasakam P, Schiestl RH. (1993) High efficiency transformation of Saccharomyces cerevisiae by electroporation. Nucleic Acids Res. Sep 11;21(18):4414-5, or the preparation of protoplasts as described in Morgan AJ. (1983) Yeast strain improvement by protoplast fusion and transformation, Experientia Suppl. 46:155-66.

The construct to be expressed is preferably cloned into a vector, in particular into plasmids which are suitable for the transformation of yeasts, such as, for example, the vector systems Yep24 (Naumovski L, Friedberg EC (1982) Molecular cloning of eucaryotic genes required for excision repair of UV-irradiated DNA: isolation and partial characterization of the RAD3 gene of Saccharomyces cerevisiae.J Bacteriol Oct;152(1):323-31), Yep13 (Broach JR, Strathern JN, Hicks JB. (1979) Transformation in yeast: development of a hybrid cloning vector and isolation of the CAN1 gene. Gene. 1979 Dec;8(1):121-33), the pRS vector series (Centromer and Episomal) (Sikorski RS, Hieter P. (1989) A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. Genetics. May;122(1):19-27) and the vector systems YCp19 or pYEXBX.

Accordingly, the invention furthermore relates to vectors, in particular plasmids comprising the above-described nucleic acids, nucleic acid constructs or expression cassettes.

The invention furthermore relates to a method for the generation of genetically modified organisms by functionally inserting, into the starting organism, an above-described nucleic acid or an above-described nucleic acid construct.

The invention furthermore relates to the genetically modified organisms, where the genetic modification

reduces the Δ22-desaturase activity and

increases the HMG-CoA reductase activity and

increases at least one of the activities selected from the group consisting of lanosterol C14-demethylase activity, squalene epoxidase activity and squalene synthetase activity

in comparison with the wild type.

In a preferred embodiment, the genetically modified organisms have a reduced Δ22-desaturase activity and an increased HMG-CoA reductase activity and an increased lanosterol C14-demethylase activity in comparison with the wild type.

In a further preferred embodiment, the genetically modified organisms have a reduced Δ22-desaturase activity and an increased HMG-CoA reductase activity and an increased squalene epoxidase activity in comparison with the wild type.

In a further preferred embodiment, the genetically modified organisms have a reduced Δ22-desaturase activity and an increased HMG-CoA reductase activity and an increased squalene synthetase activity in comparison with the wild type.

In an especially preferred embodiment, the genetically modified organisms have a reduced Δ22-desaturase activity and an increased HMG-CoA reductase activity and an increased lanosterol C14-demethylase activity and an increased squalene epoxidase activity in comparison with the wild type.

In a further, especially preferred embodiment, the genetically modified organisms have a reduced Δ22-desaturase activity and an increased HMG-CoA reductase activity and an increased lanosterol C14-demethylase activity and an increased squalene synthetase activity in comparison with the wild type.

In a further, especially preferred embodiment, the genetically modified organisms have a reduced Δ22-desaturase activity and an increased HMG-CoA reductase activity and an increased squalene epoxidase activity and an increased squalene synthetase activity in comparison with the wild type.

In a very especially preferred embodiment, the genetically modified organisms have a reduced Δ22-desaturase activity and an increased HMG-CoA reductase activity and an increased lanosterol C14-demethylase activity and an increased squalene epoxidase

activity and an increased squalene synthetase activity in comparison with the wild type.

As mentioned above, these activities are preferably increased by increasing independently, in comparison with the wild type, the gene expression of nucleic acids encoding an HMG-CoA reductase, nucleic acids encoding a lanosterol C14-demethylase, nucleic acids encoding a squalene epoxidase or nucleic acids encoding a squalene synthetase.

The furthermore preferred embodiments of the preferred genetically modified organisms according to the invention are described hereinabove in the methods.

The above-described genetically modified organisms have an increased content in ergosta-5,7-dienol and/or its biosynthetic intermediates and/or metabolites in comparison with the wild type.

Accordingly, the invention relates to an above-described genetically modified organism, wherein the genetically modified organism has an increased content in ergosta-5,7-dienol and/or its biosynthetic intermediates and/or metabolites in comparison with the wild type.

Organisms or genetically modified organisms are understood as meaning, in accordance with the invention, for example bacteria, in particular bacteria of the genus *Bacillus, Escherichia coli, Lactobacillus spec.* or *Streptomyces spec.*,

for example yeasts, in particular yeasts of the genus *Saccharomyces cerevisiae, Pichia pastoris* or *Klyveromyces spec.*,

for example fungi, in particular fungi of the genus *Aspergillus spec., Penicillium spec.* or *Dictyostelium spec.*,

and, for example, also insect cell lines which are capable of generating ergosta-5,7-dienol and/or its biosynthetic intermediates and/or metabolites, either as the wild type or owing to preceding genetic modification.

Especially preferred organisms or genetically modified organisms are yeasts, in particular of the species *Saccharomyces cerevisiae*, in particular the yeast strains *Saccharomyces cerevisiae AH22, Saccharomyces cerevisiae* GRF, *Saccharomyces cerevisiae* DBY747 and *Saccharomyces cerevisiae* BY4741.

Increasing the content in ergosta-5,7-dienol and/or its biosynthetic intermediates and/or metabolites means, for the purposes of the present invention, preferably the artificially acquired ability of an increased biosynthesis rate of at least one of these compounds mentioned at the outset in the genetically modified organism in comparison with the non-genetically-modified organism.

An increased content in ergosta-5,7-dienol and/or its biosynthetic intermediates and/or metabolites in comparison with the wild type is understood as meaning in particular increasing the content of at least one of the abovementioned compounds in the organism by at least 50%, by preference 100%, more preferably 200%, especially preferably 400% in comparison with the wild type.

The determination of the content in at least one of the abovementioned compounds is preferably carried out by analytical methods known per se and preferably relates to those compartments of the organism in which sterols are produced.

The advantage of the present invention in comparison with the prior art is as follows:

The method according to the invention makes it possible to increase the content in ergosta-5,7-dienol and/or its biosynthetic intermediates and/or metabolites in the production organisms.

The invention will now be illustrated by the examples which follow, but is not limited thereto:

I. General experimental conditions

1.Restriction

The plasmids (1 to 10 μg) were restricted in 30 μl reactions. To this end, the DNA was taken up in 24 μl of H_20 and treated with 3 μl of the buffer in question, 1 ml of BSA (bovine serum albumin) and 2 μl of enzyme. The enzyme concentration was 1 unit/μl or 5 units/μl, depending on the DNA quantity. In some cases, 1 μl of RNase was also added to the reaction in order to break down the tRNA. The restriction reaction was incubated for 2 hours at 37°C. The restriction was checked with a minigel.

2.Gel electrophoreses

The gel electrophoreses were carried out in minigel or wide minigel apparatuses. The minigels (approx. 20 ml, 8 wells) and the wide minigels (50 ml, 15 or 30 wells) consisted of 1% agarose in TAE. The running buffer used was 1 x TAE. The samples (10 μl) were treated with 3 μl of stop solution and applied. *Hind*III-cut I-DNA acted as the

standard (bands at: 23.1 kb; 9.4 kb; 6.6 kb; 4.4 kb; 2.3 kb; 2.0 kb; 0.6 kb). For the separation, 80 volts were applied for 45 to 60 minutes. Thereafter, the gel was stained in ethidium bromide solution and, under UV light, recorded with the video documentation system INTAS or photographed using an orange filter.

3.Gel elution

The desired fragments were isolated by means of gel elution. The restriction reaction was loaded into several wells of a minigel and run. Only λ-*Hin*dIII and a "sacrificial lane" were stained with ethidium bromide solution and viewed under UV light, and the desired fragment was marked. Damage by the ethidium bromide and the UV light to the DNA in the remaining wells was thus prevented. By placing the stained and the unstained gel slab next to each other, it was possible to excise the desired fragment from the unstained gel slab with reference to the marker. The agarose section with the fragment to be isolated was placed into a dialysis tube, sealed with a small amount of TAE buffer without air bubbles and placed into the BioRad minigel apparatus. The running buffer consisted of 1 x TAE, and the voltage applied was 100 V for 40 minutes. Thereafter, the polarity of the current was reversed for 2 minutes in order to redissolve the DNA which stuck to the dialysis tube. The buffer, of the dialysis tube, which contained the DNA fragments was transferred into reaction vessels and used for carrying out an ethanol precipitation. To this end, 1/10 volume of 3M sodium acetate, tRNA (1 µl per 50 µl solution) and 2.5 volumes of ice-cold 96% ethanol were added to the DNA solution. The reaction was incubated for 30 minutes at –20°C and then centrifuged for 30 minutes at 4°C at 12 000 rpm. The DNA pellet was dried and taken up in 10 to 50 µl of H_20 (depending on the DNA quantity).

4. Klenow treatment

The Klenow treatment results in DNA fragment overhangs being filled in so that blunt ends result. The following mixture was pipetted together for each µg of DNA:

DNA pellet + 11 µl H20
+ 1.5 µl 10 x Klenow buffer
+ 1 µl 0.1 M DTT
+ 1 µl nucleotide (dNTP 2 mM)
+ 1 µl Klenow polymerase (1 unit/µl)

The DNA for this purpose should originate from an ethanol precipitation in order to prevent contaminants inhibiting the Klenow polymerase. The mixture was incubated for 30 minutes at 37°C and the reaction was stopped by a further 5 minutes at 70°C. The DNA was obtained from the mixture by precipitation of ethanol and taken up in 10 µl of

H_20.

5. Ligation

The DNA fragments to be ligated were combined. The final volume of 13.1 µl contained approx. 0.5 µl of DNA with a vector:insert ratio of 1:5. The sample was incubated for 45 seconds at 70°C, cooled to room temperature (approx. 3 minutes) and then incubated for 10 minutes on ice. Thereafter, the ligation buffers were added: 2.6 µl 500 mM trisHCl pH 7.5 and 1.3 µl 100 mM $MgCl_2$, and the mixture was incubated on ice for a further 10 minutes. After addition of 1 µl 500 mM DTT and 1 µl 10 mM ATP and another 10 minutes on ice, 1 µl of ligase (1 unit/pl) was added. The whole of the treatment should be carried out as free from vibrations as possible in order not to separate joined-up DNA ends again. The ligation was carried out overnight at 14 °C.

6. E. coli transformation

Competent *Escherichia coli (E.* coli) NM522 cells were transformed with the DNA of the ligation reaction. This was accompanied by a reaction with 50 µg of the pScL3 plasmid as positive control and a reaction without DNA as zero control. For each transformation reaction, 100 µl of 8% PEG solution, 10 µl of DNA and 200 µl of competent cells *(E. coli* NM522) were pipetted into a tabletop centrifuge tube. The reactions were placed on ice for 30 minutes and shaken occasionally.

They were then given the thermal shock treatment: 1 minute at 42°C. For the regeneration, 1 ml of LB medium was added to the cells and the mixtures were incubated for 90 minutes at 37°C on a shaker. 100 µl portions of the undiluted reactions, of a 1:10 dilution and of a 1:100 dilution were plated onto LB + ampicillin plates and incubated overnight at 37°C.

7. Plasmid isolation from E. coli (miniprep)

E. coli colonies were grown overnight in 1.5 ml of LB + ampicillin medium in tabletop centrifuge tubes at 37°C and 120 rpm. On the next day, the cells were centrifuged for 5 minutes at 5000 rpm and 4°C and the pellet was taken up in 50 µl of TE buffer. Each reaction was treated with 100 µl of 0.2 N NaOH, 1% SDS solution, mixed and placed on ice for 5 minutes (cell lysis). Thereafter, 400 µl of sodium acetate/NaCl solution (230 µl of H_20, 130 µl of 3 M sodium acetate, 40 µl of 5M NaCl) were added, and the reaction was mixed and placed on ice for a further 15 minutes (protein precipitation). After centrifugation for 15 minutes at 11 000 rpm, the supernatant, which contains the plasmid DNA, was transferred into an Eppendorf tube. If the supernatant was not entirely clear, it was recentrifuged. The supernatant was treated with 360 µl of ice-cold isopropanol and incubated for 30 minutes at -20°C (DNA precipitation). The DNA was centrifuged off (15 min, 12 000 rpm, 4°C), the supernatant was discarded, and the

pellet was washed in 100 µl of ice-cold 96% ethanol, incubated for 15 minutes at -20°C and recentrifuged (15 min, 12 000 rpm, 4°C). The pellet was dried in a Speed Vac apparatus and then taken up in 100 µl of H_20. The plasmid DNA was characterized by restriction analysis. To this end, 10 µl of each reaction were restricted and separated by gel electrophoresis in a wide minigel (see above).

8. Plasmid preparation from E. coli (maxiprep)

In order to isolate larger amounts of plasmid DNA, the maxiprep method was carried out. Two flasks containing 100 ml of LB + ampicillin medium were inoculated with a colony or with 100 µl of a frozen culture containing the plasmid to be isolated and incubated overnight at 37°C and 120 rpm. On the next day, the culture (200 ml) was transferred into a GSA beaker and centrifuged for 10 minutes at 4000 rpm (2600 x g). The cell pellet was taken up in 6 ml of TE buffer. To digest the cell wall, 1.2 ml of lysozyme solution (20 mg/ml TE buffer) were added and the mixture was incubated for 10 minutes at room temperature. The cells were subsequently lysed with 12 ml of 0.2 N NaOH, 1% SDS solution and a further 5 minutes' incubation at room temperature. The proteins were precipitated by addition of 9 ml of cold 3 M sodium acetate solution (pH 4.8) and 15 minutes' incubation on ice. After the centrifugation (GSA: 13 000 rpm (27 500 x g), 20 min, 4°C), the supernatant, which contained the DNA, was transferred into a fresh GSA beaker and the DNA was precipitated with 15 ml of ice-cold isopropanol and 30 minutes' incubation at -20°C. The DNA pellet was washed in 5 ml of ice-cold ethanol and dried in the air (approx. 30-60 min). It was then taken up in 1 ml of H_20. The plasmid was verified by restriction analysis. The concentration was determined by applying dilutions to a minigel. A microdialysis (pore size 0.025 µm) was carried out for 30-60 minutes in order to reduce the salt content.

9. Yeast transformation

A preculture of the strain Saccharomyces cerevisiae AH22 was established for the yeast transformation. A flask containing 20 ml of YE medium was inoculated with 100 µl of the frozen culture and incubated overnight at 28°C and 120 rpm. The main culture was carried out under identical conditions in flasks containing 100 ml of YE medium which had been inoculated with 10 µl, 20 µl or 50 µl of the preculture.

9.1 Generation of competent cells

On the next day, the flasks were counted using a hematocytometer and the flask with a cell concentration of 3 - 5 x 10^7 cells/ml was chosen for the following procedure. The cells were harvested by centrifugation (GSA: 5000 rpm (4000 x g) 10 min). The cell pellet was taken up in 10 ml of TE buffer and divided between two tabletop centrifuge tubes (5 ml each). The cells were centrifuged off for 3 minutes at 6000 rpm and washed twice with in each case 5 ml of TE buffer. The cell pellet was subsequently

taken up in 330 µl of lithium acetate buffer per 10^9 cells, transferred into a sterile 50 ml Erlenmeyer flask and shaken for one hour at 28°C. The cells were thus competent for the transformation.

9.2 Transformation

For each transformation reaction, 15 µl of herring sperm DNA (10 mg/ml), 10 µl of DNA to be transformed (approx 0.5 µg) and 330 µl of competent cells were pipetted into a tabletop centrifuge tube and incubated for 30 minutes at 28°C (without shaking). Thereafter, 700 µl 50% PEG 6000 were added and the reactions were incubated for a further hour at 28°C without shaking. This was followed by 5 minutes' heat shock treatment at 42°C. 100 µl of the suspension were plated onto selection medium (YNB, Difco) in order to select for leucine prototrophism. In the case of selection of G418 resistance, the cells are regenerated following the heat shock treatment (see 9.3, regeneration phase).

9.3 Regeneration phase

Since the selection marker is the resistance to G418, the cells required time for expressing the resistance gene. The transformation reactions were treated with 4 ml of YE medium and incubated overnight at 28°C on a shaker (120 rpm). On the next day, the cells were centrifuged off (6000 rpm, 3 min), taken up in 1 ml of YE medium, and 100 µl or 200 µl of this were plated onto YE + G418 plates. The plates were incubated for several days at 28°C.

10. Reaction conditions for the PCR

The reaction conditions for the polymerase chain reaction must be optimized for each individual case and are not generally valid for each procedure. It is thus possible to vary, inter alia, the amount of DNA employed, the salt concentrations and the melting point. For our approach, it proved suitable to combine the following substances in an Eppendorf tube suitable for use in thermocyclers: 5 µl of Super Buffer, 8 µl of dNTPs (0.625 µM each), 5'-primer, 3'-primer and 0.2 µg of template DNA, dissolved in such an amount of water that a total volume of 50 µl for the PCR reaction results, were added to 2 µl (= 0.1 U) Super Taq polymerase. The reaction was centrifuged briefly and covered with a drop of oil. Between 37 and 40 cycles were selected for the amplification.

II. Examples

Example 1

Expression of a truncated HMG-CoA reductase in S.cerevisiae GRF

The coding nucleic acid sequence for the expression cassette consisting of *ADH*-promoter-*tHMG*-tryptophan-terminator was amplified from the vector YepH2 (Polakowski et al. (1998) Overexpression of a cytosolic hydroxymethylglutaryl-CoA reductase leads to squalene accumulation in yeast. Appl Microbiol Biotechnol. Jan;49(1):66-71) by PCR using standard methods as detailed above under the general reaction conditions.

The primers used for this purpose are the DNA oligomers AtHT-5' (forward: tHMGNotF: 5'- CTGCGGCCGCATCATGGACCAATTGGTGAAAACTG-3'; SEQ. ID. NO. 11) and AtHT-3' (reverse: tHMGXhoR: 5'- AACTCGAGAGACACATGGTGCTGTTGTGCTTC-3'; SEQ. ID. No. 12).

The resulting DNA fragment was first treated with Klenow and then cloned blunt-ended into the vector pUG6 into the EcoRV cleavage site, giving rise to the vector pUG6-*tHMG* (Figure 1).

Following the isolation of the plasmid, an extended fragment was amplified from the vector pUG-*tHMG* by means of PCR so that the resulting fragment consists of the following components: *loxP-kanMX-ADH*-promoter-*tHMG-tryptophan*-terminator-*loxP*. The primers chosen were oligonucleotide sequences which, at the 5' and 3' overhangs, comprise the 5' or the 3' sequence of the *URA3* gene, respectively, and in the annealing region the sequences of the *loxP* regions 5' and 3' of the vector pUG-*tHMG*. This ensures that firstly the entire fragment including *Kan*R and *tHMG* is amplified and secondly that this fragment can subsequently be transformed into yeast and the entire fragment integrates into the yeast *URA3* gene locus by homologous recombination.

The selection marker used is the resistance to G418. The resulting strain *S.cerevisiae GRF*-tH1ura3 is Uracil-auxotrophic and contains a copy of the gene *tHMG* under the control of the *ADH* promoter and tryptophan terminator.

In order to subsequently remove the resistance to G418 again, the resulting yeast strain is transformed with the *cre* recombinase vector pSH47 (Guldener U, Heck S, Fielder T, Beinhauer J, Hegemann JH. (1996) A new efficient gene disruption cassette for repeated use in budding yeast. Nucleic Acids Res. Jul 1;24(13):2519-24.). Owing to this vector, the *cre* recombinase is expressed in the yeast, and, as a consequence, the sequence region within the two *loxP* sequences recombines out of the gene. The result is that only one of the two *loxP* sequences and the *ADH-tHMG-TRP* cassette are retained in the URA3 gene locus. As a consequence, the yeast strain loses the G418 resistance again and is thus suitable for integrating further genes into the yeast strain by means of this cre-lox system or removing them, respectively. The vector pSH47 can

now be removed again by counterselection on YNB agar plates supplemented with Uracil (20 mg/l) and FOA (5-fluoroorotic acid) (1g/l). To this end, the cells which bear this plasmid must first be cultured under nonselective conditions and subsequently be grown on FOA-containing selective plates. Only those cells which are not capable of synthesizing Uracil themselves are capable of growing under these conditions. In the present case, these are cells which no longer contain plasmid (pSH47).

The yeast strain GRFtH1ura3 and the original strain GRF were cultured for 48 hours in WMXIII medium at 28°C and 160 rpm in a culture volume of 20 ml. 500 µl of this preculture were subsequently transferred into a 50 ml main culture of the same medium and cultured for 4 days at 28°C and 160 rpm in a baffle flask.

The sterols were extracted after 4 days following the method as described in Parks LW, Bottema CD, Rodriguez RJ, Lewis TA. (1985) Yeast sterols: yeast mutants as tools for the study of sterol metabolism. Methods Enzymol. 1985;111:333-46 and analyzed by gas chromatography. This gives the data listed in table 1. The percentages are based on the yeast dry weight.

Table 1

Sterol content [peak area/gDM]	S.cerevisiae GRFtH1ura3	S.cerevisiae GRF
Squalene	9.93	0.1
Lanosterol	0.83	0.31
Zymosterol	1.18	1.07
Fecosterol	1.10	0.64
Episterol/ergosta-5,7-dienol	1.04	0.72
Dimethyl-zymosterol	0.34	0.13

Example 2

Expression of *ERG1* in *S. cerevisiae* GRFtH1ura3 with simultaneous deletion of ERG5; generation of GRFtH1ura3ERG1erg5

Example 2.1

Generation of the integration vector pUG6-*ERG1*

The DNA sequence for the cassette consisting of ADH-promoter-*ERG1*-tryptophan-terminator was isolated from the vector pFlat3-*ERG1* by restriction with the enzymes *Nhe*I and *Bsp68*I(*Nru*I) using standard methods. The resulting DNA fragment was treated with Klenow and then cloned blunt-ended into the vector pUG6 into the *EcoR*V cleavage site, giving rise to the vector pUG6 -*ERG1* (Figure 2).

Example 2.2.

Integrative transformations

Following the isolation of the plasmid, an extended fragment was amplified from the vector pUG6-*ERG1* by means of PCR so that the resulting fragment consists of the following components: *lox*P-kanMX-*lox*P-*ADH1*-Pr.-*ERG1*-Trp-Term. The primers used were oligonucleotide sequences which contain, in the annealing region, the sequences beyond the cassette to be amplified, of the vector pUG6-*ERG1*, and at the 5' and 3' overhangs the 5' or the 3' sequence of the integration locus *ERG5*, respectively. This ensures that firstly the entire fragment including *KanR* and the target gene *ERG1* is amplified and secondly that this fragment can subsequently be transformed into yeast and integrates into the yeast target gene locus *ERG5* by homologous recombination. The following primers were used for this purpose:

ERG5-Crelox-5' (SEQ ID NO: 13): 5'-ATGAGTTCTG TCGCAGAAAA TATAATACAA CATGCCACTC CCAGCTGAAGCTTCGTACGC-3' and

ERG5-Crelox-3' (SEQ ID NO: 14): 5'-TTATTCGAAG ACTTCTCCAG TAATTGGGTC TCTCTTTTTG GCATAGGCCA CTAGTGGATC TG-3'

The selection marker used is the resistance to geneticin (G418). The resulting strain contains one copy of the target gene *ERG1* under the control of the *ADH1* promoter and the tryptophan terminator. By integration of the gene it is simultaneously possible to delete the corresponding gene *ERG5* of the target locus. In order to subsequently remove the resistance to G418 again, the resulting yeast strain is transformed with the *cre* recombinase vector pSH47. Owing to this vector, the *cre* recombinase is expressed in the yeast, and, as a consequence, the sequence region within the two *loxP* sequences recombines out of the gene, the result of which is that only one of the two *loxP* sequences and the cassette consisting of *ADH1*-prom.-*ERG1*-*TRP1*-term. are retained in the target locus *ERG5*. As a consequence, the yeast strain loses the G418 resistance again. The vector pSH47 can now be removed selectively by cultivation on FOA medium.

The resulting yeast strain GRFtH1ura3ERG1erg5 was cultured for 48 hours in WMVII medium at 28°C and 160 rpm in a culture volume of 20 ml. 500 µl of this preculture were subsequently transferred into a 50 ml main culture of the same medium and cultured for 3 days at 28°C and 160 rpm in a baffle flask.

The sterols were extracted after 4 days following the method as described in Parks LW, Bottema CD, Rodriguez RJ, Lewis TA. (1985) Yeast sterols: yeast mutants as tools for the study of sterol metabolism. Methods Enzymol. 1985;111:333-46 and analyzed by gas chromatography. This gives the data listed in table 2. The percentages are based on the yeast dry weight.

Table 2

Sterol content [peak area/gDM]	S.cerevisiae GRFtH1ura3ERG1erg5	S.cerevisiae GRF
Squalene	8.1	0.1
Lanosterol	2.42	0.31
Zymosterol	1.35	1.07
Fecosterol	2.01	0.64
Episterol/ergosta-5,7-dienol	12.21	0.72
Dimethyl-zymosterol	1.02	0.13

Comparative example 1

Deletion of ERG5 in *S. cerevisiae* GRFtH1ura3; generation of GRFtH1ura3erg5

The deletion of ERG5 in *S. cerevisiae* GRFtH1ura3 was carried out analogously to example 2. In order to delete only the ERG5 gene, the same method was used, but the vector pUG6 was employed instead of the vector pUG6-ERG1. This vector pUG6 contains no cassette consisting of ADH-prom-ERG1-Trp-term. By using this vector, it is possible to delete one gene, in this case the gene ERG5.

The resulting yeast strain GRFtH1ura3erg5 was cultured for 48 hours in WMVII medium at 28°C and 160 rpm in a culture volume of 20 ml. 500 µl of this preculture were subsequently transferred into a 50 ml main culture of the same medium and cultured for 3 days at 28°C and 160 rpm in a baffle flask.

The sterols were extracted after 4 days following the method as described in Parks LW, Bottema CD, Rodriguez RJ, Lewis TA. (1985) Yeast sterols: yeast mutants as tools for the study of sterol metabolism. Methods Enzymol. 1985;111:333-46 and analyzed by gas chromatography. This gives the data listed in table 3. The percentages are based on the yeast dry weight.

Table 3

Sterol content [peak area/g DM]	GRFtH1ura3ERG1erg5 (Example 2)	GRFtH1ura3erg5 (Comparative example)
Squalene	8.1	13.18
Lanosterol	2.42	0.78
Zymosterol	1.35	0.10
Fecosterol	2.01	1.03
Episterol/ergosta-5,7-dienol	12.21	8.98
4,4-Dimethylzymosterol	1.02	0.21